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Research Paper

Phenotypic Plasticity Determines Cancer Stem Cell Therapeutic Resistance in Oral Squamous Cell Carcinoma

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ABSTRACT

Cancer stem cells (CSCs) drive tumour spread and therapeutic resistance, and can undergo epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) to switch between epithelial and post-EMT sub-populations. Examining oral squamous cell carcinoma (OSCC), we now show that increased phenotypic plasticity, the ability to undergo EMT/MET, underlies increased CSC therapeutic resistance within both the epithelial and post-EMT sub-populations. The post-EMT CSCs that possess plasticity exhibit particularly enhanced therapeutic resistance and are defined by a $CD44^{high}EpCAM^{low/-}CD24^{+}$ cell surface marker profile. Treatment with TGFβ and retinoic acid (RA) enabled enrichment of this sub-population for therapeutic testing, through which the endoplasmic reticulum (ER) stressor and autophagy inhibitor Thapsigargin was shown to selectively target these cells. Demonstration of the link between phenotypic plasticity and therapeutic resistance, and development of an *in vitro* method for enrichment of a highly resistant CSC sub-population, provides an opportunity for the development of improved chemotherapeutic agents that can eliminate CSCs.

1. Introduction

Oral squamous cell carcinoma (OSCC) has an annual worldwide incidence of over 300,000 cases, a mortality rate of 48% (Torre et al., 2015), and commonly develops therapeutic resistance (da Silva et al., 2012). Resistance to therapeutic regimens is a major problem for cancer therapy, as it precludes complete ablation of the tumour and enables local and distant tumour recurrence, the main cause of cancer mortality. Cancer stem cells (CSCs), the sub-population of tumour cells that possess tumour-initiating potential (Clarke et al., 2006; Driessens et al., 2012), express heightened resistance to therapy compared to the majority non-stem cell population (Gupta et al., 2009; Li et al., 2008) and also drive tumour invasion and metastasis (Charafe-Jauffret et al., 2010; Hermann et al., 2007). In common with several other solid tumours, CSCs in OSCC are $CD44^{+}$ (Prince et al., 2007). However it has recently become apparent that, despite being genetically homogenous, CSCs exhibit heterogeneous phenotypes (Biddle et al., 2011; Hermann et al., 2007; Liu et al., 2014). It has further been demonstrated that CSC heterogeneity within a tumour provides a non-genetic source of variation in therapeutic response (Kreso et al., 2013), although the CSC sub-populations underlying this variation have not been elucidated.

Epithelial-to-mesenchymal transition (EMT) is a developmental process in which epithelial cells acquire a migratory mesenchymal phenotype (Hay, 2005), and is often re-activated in cancer to drive tumour invasion and metastasis (Gjerdrum et al., 2010; Yang et al., 2008). In OSCC, the secreted cytokine TGFβ is an inducer of EMT and high EMT activity correlates with poor prognosis (Jensen et al., 2015). Mesenchymal-to-epithelial transition (MET), where migratory tumour cells revert back to an epithelial phenotype, also occurs in tumours and is often required in order to enable new tumour growth at metastatic sites (Tsai et al., 2012). Therefore, a level of phenotypic plasticity that enables sequential EMT and MET is important to tumour progression (Brabletz, 2012). This phenotypic plasticity is epigenetically regulated (Chaffer et al., 2013; Ke et al., 2010), and not all cells within the epithelial and post-EMT sub-populations possess the plasticity required to enable EMT/MET (Biddle et al., 2011; Chaffer et al., 2013).

We previously identified two distinct CSC sub-populations in OSCC: $CD44^{+}$ $EpCAM^{high}$ proliferative epithelial CSCs and $CD44^{high}EpCAM^{low/-}$ migratory/metastatic post-EMT CSCs (Biddle et al., 2011). Cells were able to switch between these two sub-populations by undergoing EMT and MET. Equivalent CSC sub-populations have now also been identified in breast cancer (Liu et al., 2014; Sarrio et al., 2012). We also identified a hierarchy of plasticity within the post-EMT CSC sub-population, such that only a subset of post-EMT CSCs could undergo MET to return to an epithelial phenotype (Biddle et al., 2011).

In the present study, we sought to examine the therapeutic resistance of heterogeneous CSC sub-populations in OSCC. We found that cellular

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phenotypic plasticity intersects with EMT to determine therapeutic resistance of CSCs. We identified a sub-population of post-EMT CSCs that are highly plastic, resistant to chemotherapeutic drugs including the CSC-targeted therapy (Gupta et al., 2009) salinomycin, and exhibit a CD44-^{high}EpCAM$^{low/-}$CD24^{+} cell surface marker profile. Their high plasticity and consequent re-establishment of heterogeneity posed a challenge for therapeutic testing, and we therefore developed a co-treatment regime of TGFβ with retinoic acid (RA) for stabilization and enrichment of this sub-population. Gene expression microarray analysis identified up-regulation of processes involved in protein turnover in this sub-population, and this led us to identify Thapsigargin, a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor (Ganley et al., 2011), as a compound that selectively targets these cells. Finally, we demonstrated that fresh OSCC tumour specimens contain CSC sub-populations analogous to those identified in cell lines.

2. Materials and Methods

2.1. Cell Culture

Cell culture, including suspension culture for sphere assays, was performed as previously described (Biddle et al., 2011). The CA1 cell line was previously described (Biddle et al., 2011), and the LM cell line was recently derived in our laboratory from an OSCC of the mandibular region of the mouth. Cell removal from adherent culture was performed using 1× Trypsin–EDTA (Sigma, T3924) at 37 °C. For TGFβ and RA treatment, cells were plated at a density of 10,000 cells per ml and TGFβ and RA were added to cell culture at the indicated concentrations. Medium and TGFβ and RA were replaced every two days for at least six days and until enough cells were produced for the required assays, whilst ensuring continued sub-confluence. Floating cells in culture are greatly enriched for the post-EMT sub-population; therefore, except for when treated with TGFβ or RA, the floating cells were used at each passage of pEMT-P in order to maintain the post-EMT sub-population.

2.2. Single Cell Cloning

Single cell cloning was performed as previously described (Biddle et al., 2011), using limiting dilution and microscopic examination of wells to identify those that contain a single cell. Clonal sub-lines were maintained under standard tissue culture conditions.

2.3. Flow Cytometry and FACS

Flow cytometry was performed as previously described (Biddle et al., 2011). Antibodies for cell line staining were CD44-PE (clone G44-26, BD Bioscience), CD24-FITC (clone ML5, BD Bioscience) and EpCAM-APC (clone HEA-125, Miltenyi Biotec). For fresh tumour cells, β4-integrin-PE (Epi-P39-9B, BD Bioscience) was added and CD44-PE was replaced with CD44-PerCP/Cy5.5 (clone G44-26, BD Bioscience). Single stained controls were performed for compensation, and isotype controls were performed to set negative gating.

2.4. Immunofluorescence

FACS sorted tumour cell sub-populations were smeared onto Poly-L-Lysine slides (Thermo Scientific) and incubated at 37 °C for 30 min to promote attachment. Cells were fixed in 4% paraformaldehyde, permeabilised with 0.25% Triton-X (Sigma) in phosphate buffered saline (PBS) (Sigma, D8662), and then blocked overnight in 1% bovine serum albumin (BSA) in PBS. Cells were then incubated overnight with primary antibodies in PBS/1% BSA, washed twice in PBS/1% BSA, and then incubated for 1 h with secondary antibodies in PBS/1% BSA. Cells were washed twice in PBS/1% BSA, incubated for 1 min with DAPI (Sigma) at 1 μg/ml in PBS, washed once in PBS and then mounted with Immu-Mount (Thermo Scientific). Imaging was performed at 200× magnification, and exposure time was the same for all samples. Images were processed in Adobe Photoshop, with the threshold for high *versus* low staining the same for all samples. Antibody details can be found in the supplementary information.

2.5. RNA Extraction, cDNA Synthesis and QPCR

RNA extraction, cDNA synthesis and QPCR were performed as previously described (Biddle et al., 2011). Primer sequences are listed in the supplementary information.

2.6. Drug Dose Response Assays

Cells were plated at 1000 cells per well in flat-bottomed 96-well tissue culture plates (Corning). 24 h later, drugs were added at 4 different concentrations in triplicate technical replicates, with triplicate untreated control wells. 72 h after drug addition, cells were fixed in 4% paraformaldehyde and washed in PBS. For automated microscope analysis, cells were permeabilised with 0.1% Triton-X (Sigma) in PBS, then stained with CellMask deep red (Life Technologies H32721, used at 1:30,000 dilution) and 1 μg/ml DAPI (Sigma) for 1 h. Cells were washed twice with PBS. Cell images were acquired using an InCell 1000 automated microscope (GE), and then analysed using InCell Developer Toolbox software (GE) to determine the number of cells. Data was averaged for the triplicate technical replicates and normalized to the untreated wells. Results from at least three independent biological repeat experiments were entered into Graph-Pad Prism software to determine the dose response curve, IC50 and 95% confidence intervals for the IC50, using the nonlinear regression analysis of log(inhibitor) *versus* response with a variable slope. Drug details can be found in the supplementary information.

2.7. Microarray Analysis

RNA was extracted using the RNeasy microkit (Qiagen) and analysed using an Illumina Human HT-12 v4 gene expression array. The results were analysed using the GenomeStudio software (Illumina), with quantile normalization and a false discovery rate filter of 5% in differential expression analysis. The top 150 differentially expressed genes from each analysis were analysed with the functional annotation clustering tool on the DAVID database (Huang da et al., 2009a, 2009b). Microarray data are deposited in the GEO database under the accession numbers GSE74578 and GSE74580.

2.8. Transplantation Into Immunodeficient Mice

NOD/SCID mice were obtained from Jackson Laboratories. Mice used in this study were of mixed gender and older than 6 weeks of age. The mice were maintained in a certified isolation facility under a pathogen free environment with standard 12/12 h day and night cycle, in accordance with European guidelines. All animal procedures were approved by the Norwegian Animal Research Authority. Cells were harvested from adherent culture and resuspended in 50 μl of Matrigel (BD Biosciences) on ice. The suspension was injected orthotopically into the tongues of NOD/SCID mice. Tumours were detected by palpation and the tumour volume was manually assessed with a digital calliper.

2.9. Isolation of Cells From Human Tumours

Tumour specimens were obtained from the pathology department at Barts Health NHS Trust, with full local ethical approval and patients' informed consent. Specimen site was selected to avoid both the tumour margin and necrotic core, and specimens were kept overnight at 4 °C in epithelial growth medium (termed FAD) with 10% FBS (Locke et al., 2005). Specimens were washed in PBS to remove blood, minced into approximately 1 mm^3 pieces using scalpels, and then incubated with